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Production of biologically active hirudin in plant seeds using oleosin partitioning

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Abstract

A plant oleosin was used as a 'carrier' for the production of the leech anticoagulant protein, hirudin (variant 2). The oleosin-hirudin fusion protein was expressed and accumulated in seeds. Seed-specific expression of the oleosin-hirudin fusion mRNA was directed via an *Arabidopsis* oleosin promoter. The fusion protein was correctly targeted to the oil body membrane and separated from the majority of other seed proteins by flotation centrifugation. Recombinant hirudin was localized to the surface of oil bodies as determined by immunofluorescent techniques. The oleosin-hirudin fusion protein accumulated to ca. 1% of the total seed protein. Hirudin was released from the surface of the oil bodies using endoprotease treatment. Recombinant hirudin was partially purified through anion exchange chromatography and reverse-phase chromatography. Hirudin activity, measured in anti-thrombin units (ATU), was observed in seed oil body extracts, but only after the proteolytic release of hirudin from its oleosin 'carrier'. About 0.55 ATU per milligram of oil body protein was detected in cleaved oil body preparations. This activity demonstrated linear dose dependence. The oleosin fusion protein system provides a unique route for the large-scale production of recombinant proteins in plants, as well as an efficient process for purification of the desired polypeptide.

Introduction

Plant-based expression systems have recently received attention as alternative hosts for the production of recombinant proteins and peptides [19]. The ease by which DNA sequences may be transferred and stably integrated into the genome of many plant species [15] and the relatively low cost of plant cultivation has heightened interest in this approach. Transgenic plants have been used as vehicles for the production of a variety of different recombinant proteins. Hiatt and colleagues [12] produced two lines of tobacco transformants which, upon crossing, produced functional, correctly assembled recombinant gamma-kappa complexes that accumulated to 1.3% of the total leaf protein. Plant-based expression systems have also been used successfully to produce human serum albumin in potato tubers [35]. The recombinant proteins were targeted for secretion into the apoplastic space by addition of signal sequences derived from the extracellular PR-S protein of tobacco or from the human prepro-sequence.

Recently, the expression of recombinant proteins in seeds has become the focus for plant-based expression studies. Seeds possess a number of characteristics which make them highly suitable for this type of application. Plant seeds represent an extremely inexpensive source of protein. For example, oilseed rape seed, which is ca. 40% protein by weight, costs ca. $250 per metric tonne to produce [8]. Upon maturation, seeds lose greater than 95% of their water content [10], therefore seed proteins are already in a very concentrated form. In addition, seeds have low hydrolytic activity [42], minimizing protein degradation, and seeds can be stored for long periods of time without deterioration.

The cost of purification of recombinant proteins or peptides has generally been considered a limiting factor for the commercial application of seed-based expression systems. Pen *et al.* [29] circumvented the need to purify recombinant proteins produced in seeds by expressing phytase as an additive in animal feed. However, there are relatively few proteins which can be used in an unpurified form in this manner.

Another example of a pharmaceutically active peptide produced in plants is Leu-enkephalin, which was produced in *Brassica napus* and *Arabidopsis* seeds as an internal fusion with the storage protein napin [39]. The biological activity of the enkephalin produced was not tested. Hoffman *et al.* [13] showed that modification of the seed storage protein, phaseolin, caused retention of the recombinant form within the ER and/or Golgi, possibly due to a change in the secondary or tertiary structure of the protein, preventing its successful transport. Thus, seed storage proteins are unlikely to be suitable 'carriers' of larger peptide sequences. In addition, extraction and purification of recombinant proteins made as seed storage protein fusions is impractical for large-scale peptide production [39]. If plant systems are to become viable alternatives for the large-scale production of recombinant proteins, methods must be developed to ensure ease of purification and high yield of the recombinant protein.

An alternative to the use of seed storage proteins as 'carriers' for recombinant proteins is the exploitation of another class of seed-specific proteins called oleosins. Oleosins are small, abundant proteins embedded in the phospholipid monolayer of oil bodies [16]. In oilseed rape, they represent ca. 8–20% of the total seed protein [16, 27] and are inducible by osmotic stress, jasmonic acid, and abscisic acid [30, 38, 41]. One of the most attractive features of oleosins is their ease of purification. Oil bodies and associated proteins are easily separated from other seed components as an immiscible 'fat pad' by flotation centrifugation. Since the amino acid sequence of oleosins from different plant species diverges at their amphipathic N- and C-terminal domains [16], we postulated that recombinant proteins might be fused to oleosins without adversely affecting their targeting. Recently, it was demonstrated that an oleosin-β-glucuronidase fusion protein under the control of an *Arabidopsis* oleosin promoter was correctly targeted to the oil body membrane [36]. The correct targeting of the GUS protein (67 kDa) fused to the C-terminal end of the entire oleosin coding sequence suggests that oleosin fusions might be appropriate vehicles for expression of high-value (e.g. pharmaceutical) peptides in plant seeds. As an example of a potentially useful pharmaceutical protein, we investigated the properties of hirudin.

Hirudin, produced in the salivary glands of the leech, *Hirudo medicinalis* [23], is the most potent and specific thrombin inhibitor known (K_i = 6.3 $\times$ 10^{-11}M [4]). This protein is being studied in clinical trials for use as a blood anticoagulant (reviewed in [24]). Hirudin cDNAs have been cloned and expressed to produce biologically active protein in *Escherichia coli* [11], yeast [31], baculovirus-infected insect cells [1] and *Streptomyces lividans* [2]. Hirudin is an attractive model to evaluate the use of oleosins as 'carriers' of high-value proteins due to its low molecular weight, minimal processing requirements, and relative hydrophilicity. In addition, its antithrombin activity is easily measured by a colorimetric assay [4].

In this investigation, a fusion of an *Arabidopsis* oleosin promoter and coding sequence followed, in-frame, by a sequence encoding synthetic hiru-

din variant 2 [11], was constructed. To facilitate purification of recombinant hirudin, a sequence encoding a Factor Xa/clostripain cleavage site was interposed between the oleosin and hirudin coding sequences. This fusion system was used to evaluate the possibility of producing hirudin in seeds of the common oilseed crop, *Brassica napus* (oilseed rape). A further objective was to determine the biological activity of the recombinant protein once released from its oleosin 'carrier'.

Materials and methods

Constructs

A synthetic cDNA encoding hirudin variant 2 (HV2) was designed from the amino acid sequence [11] but employing *Brassica napus* [21] and *Arabidopsis* [37] codon usage. Four overlapping oligonucleotides were used in PCR amplification to generate a 222 bp fragment. This fragment contained a region corresponding to the hirudin coding sequence, a region encoding a proteolytic cleavage site at the 5′ end, and restriction sequences at both ends (Fig. 1A). DNA sequence analysis of the fragment was performed by the dideoxy chain-termination method [33] after end-filling and subcloning into the *Sma*I site of a pUC 19 plasmid vector. This plasmid was called pHIR.

The lambda genomic clone containing an *Arabidopsis* oleosin promoter and coding sequence [37] was used as a template in polymerase chain reactions. The oligonucleotide GVR10 (5′-**CA-*CTGCAG*GAACTCTCTGGTAAGC**-3′), which is homologous to sequences −838 to −814 (bold type) of the upstream *Arabidopsis* oleosin sequence, but modified to contain a *Pst* I site (underlined), and the oligonucleotide GVR11 (5′-CCGTCGACTTACTTGTCGTTAGATTCTT-CTCCCTGAACTCTCCCTTcgatcg**CAGTAGT-GTGCTGGCCACC**-3′) which is homologous to the 3′-coding region of the *Arabidopsis* oleosin coding region (bold type), but modified to contain a *Sal* I (underlined) and a *Pvu* I (lower case) site, were generated. These primers were used in a 30-cycle PCR amplification of the lambda clone

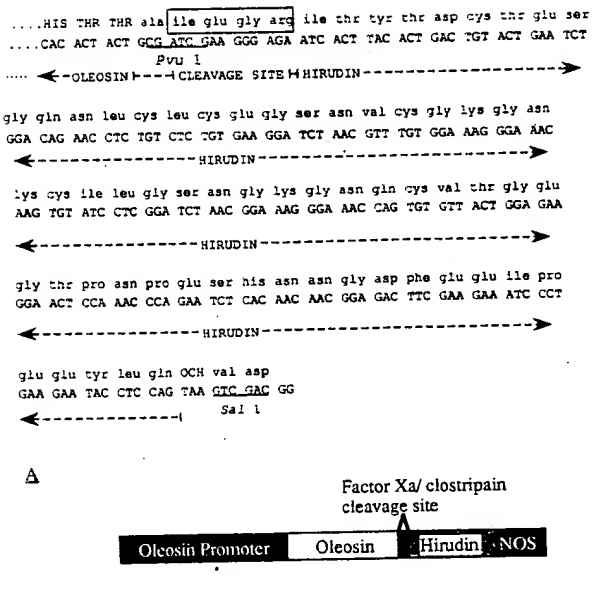

Fig. 1. Oleosin-hirudin fusion gene construct. A. Nucleotide and deduced amino acid sequence of the C-terminal end of the fusion construct. Shown are the last three amino acids of an *Arabidopsis* oleosin coding sequence, followed by an in-frame translational fusion of a proteolytic cleavage site (boxed) and the coding region of hirudin variant 2. The hirudin cDNA is flanked by *Pvu* I and *Sal* I restriction sites (underlined). B. Construct introduced into *Brassica napus* via *Agrobacterium*-mediated transformation. The oleosin-hirudin fusion gene is under the control of an *Arabidopsis* oleosin promoter (838 bp), and is terminated by a 267 bp nopaline synthase transcriptional terminator (NOS).

(annealing temperature of 45 °C). The PCR reaction mixture consisted of 16 μl dNTPs (1.25 mM), 10 μl 10× PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin), 5 μl (20 mM) of each primer, 1 μl *Taq* DNA polymerase (1 unit/μl), 1 μl (1 ng/μl template), and 64 μl H_2O. A 1652 bp fragment containing a 0.84 kb *Arabidopsis* oleosin promoter and 0.8 kb coding region was isolated, end-filled, kinased, and cloned into the *Sma* I site of pUC19. This plasmid was called pOBIL. Next, pBI121 (Clontech), containing a nopaline synthetase (nos) terminator sequence flanked by 5′ *Sal* I, 3′ *Eco* RI restriction sites was digested with these enzymes and subcloned into the *Sal* I/*Eco* RI sites of pUC19. This plasmid was called pTERM. The nos terminator was isolated from this plasmid by

cleavage with *Sal* I and *Eco* RI restriction enzymes and ligated to the 3′ end of pOBIL plasmid at the *Sal* I/*Eco* RI sites. This plasmid was called pOBILT. The hirudin fragment of pHIR was excised with *Pvu* I and *Sal* I and ligated into these sites of the pOBILT plasmid. This plasmid was called pOBHIRT. This oleosin-hirudin fusion gene of pOBHIRT was subcloned into the *Hind* III/*Eco* RI site of pBluescript KS + to generate appropriate restriction sites at the 5′ and 3′ ends, then subcloned into pCGN 1559 [25] at the *Pst* I site. This plasmid was designated pCGN-OBHIRT. The fusion gene construct is shown in Fig. 1B.

Plant transformation and analysis

The pCGN-OBHIRT construct was introduced into *Agrobacterium tumefaciens* strain EHA 101 [14] by electroporation [7]. The transformation of *Brassica napus* (cv. Westar) petioles was as described by Moloney *et al.* [26].

Recombinant DNA techniques

All standard recombinant DNA techniques (DNA digestion by restriction endonucleases, T4-DNA ligase-mediated ligations, plasmid preparations, growth of bacterial cultures) were performed according to Sambrook *et al.* [32].

Northern blotting and hybridization

RNA extractions from plant tissues were performed according to Verwoerd *et al.* [40]. Quantification of the RNA extracts was achieved through spectrophotometric measurements at OD_{260nm}. A 35 μg portion of RNA was denatured, fractionated by 1.2% formaldehyde/agarose gel electrophoresis, and transferred onto Hybond N filters (Amersham) according to manufacturers' instructions. RNA was fixed onto the membranes by UV irradiation. The filters were prehybridized in 25 ml hybridization buffer (5 × SSPE is 0.9 M NaCl, 50 mM sodium phosphate pH 7.7, 0.5 mM Na_2EDTA, 50% formamide, 5 × Denhardt's solution, 10% dextran sulfate (w/v), and 1 mg denatured herring sperm DNA) overnight. Hybridizations were performed at 42 °C with hirudin DNA labeled with α^{32}P-dCTP in fresh hybridization buffer. Filters were washed twice in 5 × SSPE at 42 °C for 15 min each.

Isolation and extraction of seed proteins

Seed proteins were isolated from 0.5 g dry (mature) seeds by grinding in 8 ml extraction buffer (0.6 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 0.15 M tricine pH 7.5). This total seed protein was fractionated into water-soluble, sedimenting, and the oil body phases by overlaying with ca. 5 ml of flotation buffer (extraction buffer containing 0.4 M sucrose instead of 0.6 M) and centrifuging at 15 000 × *g* for 1 h. Insoluble seed proteins and oil bodies were isolated, resuspended and centrifuged (washed), as above, three times. The soluble seed proteins were centrifuged three more times to remove remaining insoluble fractions. For the fractionation studies, the volumes of total seed protein and fractionated seed proteins were equalized (13 ml each). A 30 μg (3.2 μl) portion of wild-type and transformed total seed proteins were loaded onto a SDS-polyacrylamide gel. Oil bodies, soluble and resuspended insoluble seed protein fractions (3.2 μl each) were also loaded onto the gel. Protein concentration was quantitated by the BioRad protein assay using bovine serum albumin (BSA) as a standard.

Protein gel electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of seed protein extracts were performed according to Laemmli [20]. Proteins were transferred from polyacrylamide gels onto PVDF membranes in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol) at 300 mA (constant current) for 2–3 h. Seed proteins were transferred onto PVDF membrane (pore size

0.45 μm) (Millipore). Samples containing free hirudin were electrophoresed through tricine-SDS polyacrylamide gels (16.5%) [34] and fixed in 50% trichloroacetic acid prior to staining. Alternatively, fixed gels were equilibrated in transfer buffer containing 0.1% SDS and electroblotted onto Immobilon-P^{SQ} (Millipore), which has a pore size of 0.1 μm and an enhanced protein binding capacity, particularly for smaller peptides. All immunoblots were blocked with 10% skimmed milk in TBS buffer and rinsed with TBS containing 0.3% Tween-20. Rabbit anti-19 kDa *B. napus* oleosins antibodies were kindly provided by Jo Ross at John Innes Centre, UK. Mouse anti-hirudin monoclonal antibodies were generously donated by C. Koch and K. Gerner-Smidt [18]. Alkaline phosphatase (AP)-conjugated secondary antibodies recognizing rabbit and mouse IgG were purchased from BioRad and Boehringer-Mannheim, respectively. Membranes were incubated with primary antibodies for 3 h at room temperature. Membranes were incubated with secondary antibodies for 1 h. Color development of AP was according to ProtoBlot Western Blot AP system.

Proteolytic digestion of oleosin-hirudin fusion proteins and determination of anti-thrombin activity

Oil bodies were isolated as described above, then washed twice in cleavage buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$). Oil bodies were digested overnight at 25 °C in the presence or absence of Factor Xa (FXa) (New England Biolabs) or clostripain (Sigma). Factor Xa (2.5 units) or clostripain (1 unit) was added per mg oil body protein in cleavage buffer. Oil bodies were cleaved with clostripain under reducing conditions (2.5 mM DTT, final concentration). After FXa treatment, the oil body preparations (17 mg/ml) were centrifuged and the buffer underneath the fat pad (unternatant) was assayed for anti-thrombin activity. After clostripain treatment, 500 μl of the oil body preparations (14 mg/ml) were precipitated overnight at −20 °C with 11 volumes of acetone. Precipitated proteins were washed once with cold acetone, air-dried slightly, and resuspended in 500 μl of cleavage buffer. Thrombin from human plasma (Sigma) was added to assay buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$) to a concentration of 0.12 to 0.14 units/ml. This reaction buffer was stored on ice until needed. One ml of reaction buffer was added to buffer or protein extracts from cleaved or uncleaved oil body preparations and allowed to incubate at 25 °C for 5–10 min. After about 10 min, 50 pmol of the chromogenic substrate, p-tosyl-gly-pro-arg-p-nitroanilide (Sigma) was added to the above mixture. The change in absorbance at OD_{405nm} was monitored over 2 min. All samples were assayed in triplicate. The average change of absorbance per minute and the standard error of the mean was calculated and converted into units of thrombin by reference to a thrombin standard curve. Dose-dependent assays were analyzed by a least significant difference (LSD) test at the 1% level of significance.

Immunofluorescent localization of hirudin

Anti-hirudin monoclonal antibodies were used to confirm the presence of hirudin of the surface of lipid bodies. Oil body proteins from transformed *B. napus* seeds were washed three times in buffer (described above). After the third centrifugation step, the oil bodies were isolated and resuspended in cleavage buffer (described above) and separated into 200 μl (27 mg/ml) aliquots. In order to reduce non-specific staining, the oil bodies were washed three times in 300 μl PBS containing 1% BSA. The preparations were incubated with anti-hirudin monoclonal antibodies (1:20 dilution) for 6 h at 4 °C. The antibodies were diluted in PBS containing 0.1% BSA. After incubation, the oil bodies were washed three times in PBS containing 0.1% BSA. FITC-conjugated anti-mouse secondary antibodies (1:60 dilution, Sigma cat. no. F 8771) were added and incubated at room temperature for 1 h. Following this, the oil bodies were washed three times in PBS. Twenty microliters of oil body and 20 μl of mounting media

were placed on a slide. The mounting media contained a saturated solution of n-propyl gallate, in PBS. Slides were examined using a Leica Aristoplan fluorescence microscope with the standard FITC exciter and barrier filters (Filter system I3, Leica Canada, Toronto). The image was captured on Ilford FP4 film (ASA 400). A fixed exposure time was used to photograph all treatments.

A number of control treatments were performed in parallel to the antibody staining of hirudin: (1) Factor Xa was used to cleave hirudin from the surface of the oil bodies; (2) mouse serum (Sigma cat. no. M-5905) was substituted for anti-hirudin antibodies; (3) the oil bodies were stained with the fluorescent secondary antibodies to check for non-specific binding; (4) parallel experiments were conducted using oil bodies from wild-type (WT) seeds. Hirudin was cleaved from the oil bodies by addition of 10 μl of Factor Xa (1 μg/μl) and incubation at room temperature overnight with gentle shaking. The oil bodies were subsequently washed with PBS containing 1% BSA and stained with the secondary antibodies as outlined above. All experiments were conducted twice.

Purification and analysis of recombinant hirudin

The unternatant fraction from oil bodies cleaved with Factor Xa was loaded onto an FPLC Mono Q HR 5/5 anion exchange column (Pharmacia) pre-equilibrated with buffer A (20 mM N-methyl piperazine pH 4.7) and the column was washed with this buffer until the A_{280} returned to baseline. Proteins were eluted with 20 ml of a 0–35% gradient of buffer B (buffer A + 0.5 M $(NH_4)_2$ SO_4) and collected in 1 ml fractions. Fractions exhibiting anti-thrombin activity were pooled and desalted on PD-10 columns (Pharmacia) equilibrated with water.

Pooled fractions from anion exchange chromatography were loaded onto a Vydac C_{18} TP1022 semipreparative reversed-phase column (Separations Group) equilibrated with 0.1% trifluoroacetic acid. The column was washed with 7% acetonitrile, 0.1% trifluoroacetic acid and developed with a 7–30% gradient of acetonitrile, 0.1% trifluoroacetic acid. Fractions exhibiting anti-thrombin activity were dried on a Speed-Vac and separated on 16.5% tricine-SDS polyacrylamide gels as described above.

Results

Expression of oleosin-hirudin fusion gene in Brassica napus

An in-frame fusion of an *Arabidopsis* oleosin coding region, followed by that encoding hirudin variant 2 was made. This fusion gene was placed under the control of an *Arabidopsis* oleosin promoter and was terminated by a nopaline synthetase transcriptional terminator. This construct (Fig. 1B) was introduced into *Brassica napus* (oilseed rape) by *Agrobacterium*-mediated transformation. Southern blot analysis of kanamycin resistant plants identified a number of transformants which had incorporated the oleosin-hirudin fusion gene into their genome (an average of 2–3 copies per genome). These transformants were screened for the expression of the oleosin-hirudin mRNA. RNA was extracted from developing seeds (mid-cotyledonary stage) of transformed and wild type *B. napus* plants. Expression of the oleosin-hirudin mRNA was observed on northern blots probed with radiolabelled hirudin-encoding cDNA. As shown in Fig. 2, hirudin gene expression occurred in three of the four transformants tested. The hirudin DNA probe did not hybridize to seed RNA from wild-type plants. Northern blot analysis of RNA from leaf, stem, bud and embryo tissue of transformed plants showed that expression of the oleosin-hirudin fusion RNA was embryo-specific (data not shown). This observation is consistent with previous work using this oleosin promoter [30].

Detection and localization of oleosin-hirudin fusion protein

Seed proteins can be separated into three distinct fractions when the seeds are ground in an aque-

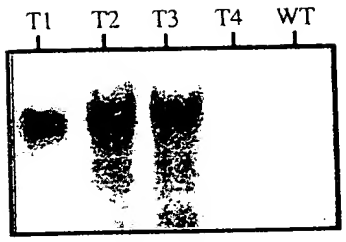

Fig. 2. Northern blot demonstrating expression of the oleosin-hirudin fusion gene in oilseed rape embryos. A 35 μg portion of total RNA from mid-cotyledonary embryos derived from wild-type (WT) and four different *B. napus* transformants (T_1-T_4) was loaded onto a 1.2% agarose gel containing 6% formaldehyde. The gel was run overnight at 20 V and the RNA blotted onto Hybond N. The membrane was probed with ^{32}P-labelled hirudin cDNA (10^6 cpm per ml hybridization buffer).

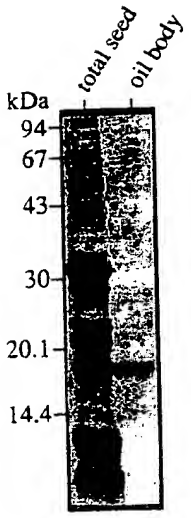

Fig. 3. Oleosin enrichment from total seed protein via floatation centrifugation. Total seed protein and oil body protein was obtained from an equal weight of seed (0.5 g) from plants transformed with the oleosin-hirudin fusion gene. The final volume of total seed protein and oil body protein obtained were equalized (13 ml). Equal volumes from both seed preparations (5.4 μl each) were loaded and electrophoresed via SDS-PAGE (15% gel). The resulting gel was stained with Coomassie blue.

ous buffer and centrifuged. In *Brassica napus*, these fractions consist of soluble proteins (mainly the storage proteins, napin and cruciferin), insoluble (pelleted) proteins, and an immiscible 'fat pad' consisting of oil body proteins. As a result of their hydrophobic nature, seed protein extracts consisting primarily of oil bodies and associated proteins can be easily recovered by flotation centrifugation. This method involves the repeated isolation, resuspension, and centrifugation (washing) of oil bodies. Dry seeds from a *B. napus* transformant expressing the oleosin-hirudin fusion gene were ground in extraction buffer, overlaid with flotation buffer, and centrifuged (see Materials and methods). The three fractions were isolated and washed, and their volumes equalized (see Materials and methods). Total seed protein from wild-type and transformed plants was isolated by grinding dry seeds in extraction buffer. As shown in Fig. 3, this extraction procedure separated the oil body proteins, typified by the 19 kDa oleosin of *B. napus* [28] from the majority of the other seed proteins. Western blot analysis was used to determine the localization of the oleosin-hirudin fusion protein in seed extracts. Aliquots of the total, soluble, resuspended insoluble, and oil body fractions from the transformed (T) plant seeds and total seed protein from wild-type (WT) plants, were loaded onto an SDS-polyacrylamide gel. These gels were used in immunoblotting experiments by probing with anti-hirudin monoclonal antibodies. The oleosin-hirudin fusion protein has an expected molecular mass of 26 kDa. As shown in Fig. 4, the fusion protein detectable by anti-hirudin antibodies was preferentially localized to the oil body fraction (T, OBP). A small amount of the oleosin-hirudin fusion protein was also detected by these antibodies in the soluble protein fraction. This may be due either to the difficulty associated with complete removal of oil bodies from the soluble seed protein fraction, or the disruption of oil bodies and subsequent release of oleosins during preparation. Whenever oleosin-hirudin fusion proteins were detected immunologically in seed fractions other than the fat pad, they were always in the presence of native oleosin proteins. The anti-hirudin antibodies did not recognize any protein from wild-type total seed protein (WT, total).

It was expected that the oleosin-hirudin fusion

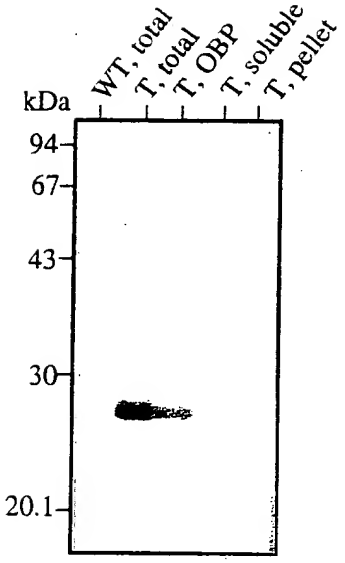

Fig. 4. Immunological determination of localization of oleosin-hirudin fusion protein by fractionation of seed proteins. Total seed protein (total), oil body protein (OBP), soluble, or insoluble (pellet) seed protein was extracted from equal amounts of transformed (T) *Brassica napus* seeds (see Materials and methods). Total seed protein from wild-type (WT) seeds was also extracted. After equalizing the volumes of all fractions, identical volumes of protein were electrophoresed via SDS-PAGE (12% acrylamide gel), blotted onto PVDF membrane, and probed with anti-hirudin antibodies. Note the absence of antibody binding to proteins derived from wild-type seed or from the transformed seed pellet fraction.

protein would be recognized by both anti-hirudin and anti-oleosin antibodies. To prove this, total seed protein from transformed and wild-type seeds was transferred onto two sets of membranes. One set of membranes was probed with anti-oleosin antibodies, the other with anti-hirudin antibodies. As seen in Fig. 5, panel A, a band corresponding to a molecular mass of ca. 26 kDa was recognized by the anti-hirudin antibody in transformed (T), but not wild type (WT) oil body proteins. When a membrane with these identical proteins was probed with anti-oleosin antibodies (Fig. 5, panel B), native oleosins, having a molecular weight of 19–24 kDa, were recognized in both plant types. However, an additional band in transformed oil bodies (indicated by the arrow), and corresponding to a band of identical molecular weight recognized by the anti-

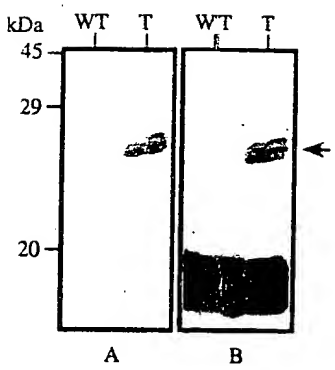

Fig. 5. Immunological detection of fusion protein with hirudin and oleosin antibodies. A 50 μg portion of each of total seed protein from wild-type (WT) or transformed (T) seeds was subjected to SDS-PAGE, transferred onto PVDF membrane, and probed with either anti-hirudin (A) or anti-oleosin (B) antibodies. Note the presence of a ca. 26 kDa protein (arrow) in the transformed seed proteins probed with either antibody. This band is absent in wild-type seed protein.

hirudin antibodies (panel A), was recognized by anti-oleosin antibodies.

Immunofluorescence localization of hirudin

Oil bodies from transformed seeds were isolated and probed with anti-hirudin primary antibodies. Antibody binding to oil bodies was detected by probing with FITC-conjugated secondary antibodies and visualization with fluorescence microscopy. As shown in Fig. 6, transformed oil bodies (Fig. 6C) showed significant fluorescence as a result of this treatment (Fig. 6D). To verify that antibody binding was due to the presence of hirudin on the surface of oil bodies, the oil bodies (Fig. 6E) were treated with Factor Xa. Oil body fluorescence was significantly reduced after proteolytic treatment (Fig. 6F), although low levels of residual fluorescence were observed. This is probably a result of incomplete cleavage of hirudin from its oleosin 'carrier'. Untransformed oil bodies (Fig. 6A) showed no fluorescence when probed with the above primary and secondary antibodies (Fig. 6B). No fluorescence was observed when transformed oil bodies were probed with mouse serum and secondary antibodies, or secondary antibodies, alone (data not shown).

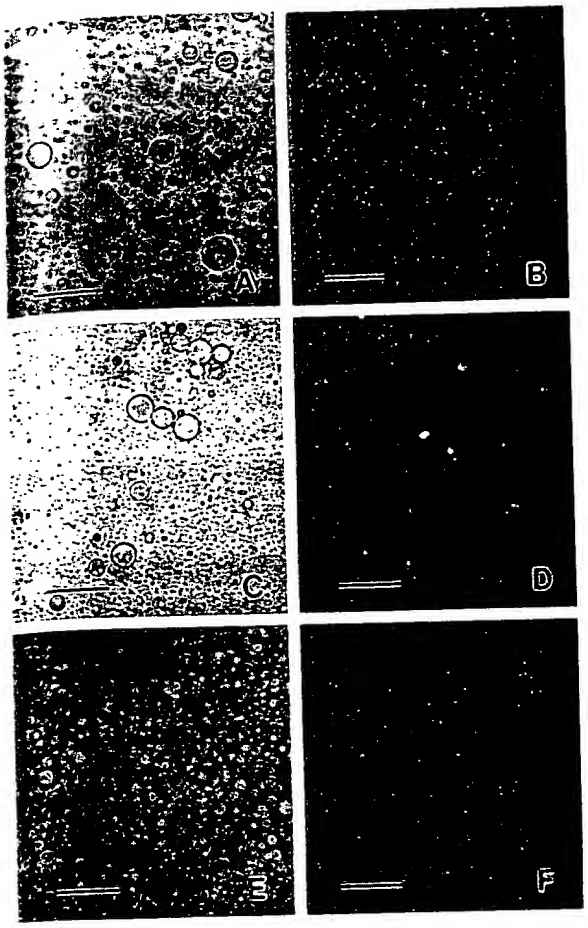

Fig. 6. Immunofluorescence localization of hirudin. Oil bodies from transformed and wild-type seeds were isolated and probed with anti-hirudin monoclonal antibodies and the appropriate FITC-conjugated secondary antibodies (see Materials and methods). A, C, and E. Light microscopy. B, D, and F. Fluorescence microscopy. Transformed oil bodies were treated (E, F) or untreated (C, D) with Factor Xa. As shown, oil body fluorescence is significantly reduced after Factor Xa treatment. Wild-type oil bodies (A) demonstrated no fluorescence (B). Scale bar = 25 μm.

Enzymatic assay of hirudin activity

Hirudin activity is measured using a colorimetric thrombin inhibition assay. This assay measures the rate of thrombin-mediated proteolysis of a chromogenic substrate (p-tosyl-gly-pro-arg-nitroanilide) that, upon cleavage, absorbs light at 405 nm. *B. napus* oil bodies from wild-type and transformed seeds were analyzed for anti-thrombin activity. Transformed and wild-type oil bodies were treated in the presence or absence of Factor Xa overnight at room temperature. Thrombin was added to the unternatant isolated from these samples, or to buffer, alone. The thrombin proteolytic activity present in each mixture, as measured by the increase in absorbance at 405 nm, was determined for each sample. As seen in Fig. 7, the activity of the thrombin was unchanged by addition of unternatant from wild type oil bodies (either cleaved with FXa or uncleaved). Significantly, oil body proteins from seeds transformed with the oleosin-hirudin fusion gene also demonstrate no anti-thrombin activity prior to Factor Xa treatment. Anti-thrombin activity in transformed plants only occurred after the hirudin was released from the oleosin-hirudin fusion by specific proteolytic digestion. The hirudin activity released corresponds to ca. 0.55 anti-thrombin units per mg transformed oil body protein cleaved with Factor Xa. Treatment of oil

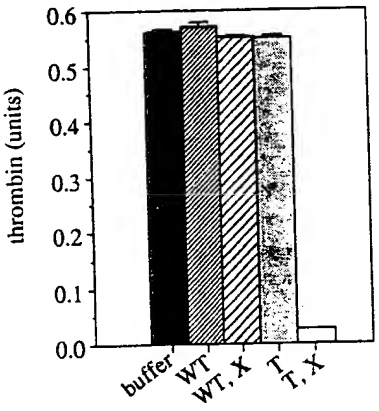

Fig. 7. Measurement of anti-thrombin activity in transformed and wild-type oil body extracts. Oil bodies from transformed (T) and wild-type (WT) seeds were treated in the presence (X) or absence of Factor Xa. The undernatant from these samples, or buffer alone, was added to thrombin in buffer. The change in thrombin activity was measured per mg of oil body extract added. In the case of the buffer control, as no protein was present, the activity was normalized by dividing by the average protein content of the other samples (all of which were approximately the same). The thrombin activity present in the samples cleaved with Factor Xa have been adjusted to account for the presence of contaminating thrombin present in commercial Factor Xa. As shown, thrombin activity is only reduced when unternatant from Factor Xa-treated, transformed oil bodies (T, X) is added.

body proteins with the protease clostripain (an inexpensive enzyme which cleaves after arginine residues) showed similar results as those obtained with Factor Xa cleavage. As shown in Table 1, addition of 20, 40, and 60 μl of oil body proteins from wild type oil bodies treated or untreated with clostripain, or from untreated oil bodies of transformed seeds had no effect on thrombin activity. However, addition of protein extracts from transformed oil bodies cleaved with clostripain (Table 1; TF + clostripain) resulted in a dose-dependent inhibition of thrombin activity. In this assay, ca. 0.2 anti-thrombin units per mg transformed oil body protein cleaved with clostripain was measured. The lower levels of anti-thrombin activity (per mg of oil body protein) obtained from clostripain-treated oil bodies compared to FXa-treated oil bodies is likely due to the nature of protein preparation. Clostripain is only enzymatically active in the presence of 2.5 mM DTT. Optimal anti-thrombin activity of hirudin is dependent on the formation of three intramolecular disulfide bridges [4]. As a result, care must be taken in order to remove the DTT from samples incubated with this reducing agent. On account of these technical hurdles, all other cleavages reported here employed Factor Xa, despite its relative high cost compared with clostripain.

Table 1. Determination of dose-dependent inhibition of thrombin activity by wild-type and transformed oil body extracts. Oil body proteins from transformed (TF) and wild-type (WT) extracts were digested in the presence or absence of clostripain. Acetone-precipitated proteins were resuspended in cleavage buffer (see Materials and methods) and added in 20, 40, or 60 μl volumes to thrombin in buffer. As a control, 20, 40, or 60 μl of cleavage buffer, alone, was added to thrombin in this buffer. Thrombin activity, in thrombin units, is reported. All assays were performed in triplicate. Least significant difference (LSD) tests separated data into statistically similar or different groupings, shown as superscripts. Significant anti-thrombin activity was only observed in transformed oil body proteins treated with clostripain. This activity was dose-dependent.

Sample	Thrombin activity		
	20 μ	40 μl	60 μl
Buffer	0.1368^{ab}	0.1290^{bcd}	0.1222^{d}
WT	0.1348^{abc}	0.1339^{abc}	0.1262^{cd}
WT + clostripain	0.1378^{a}	0.1382^{a}	0.1330^{abc}
TF	0.1402^{a}	0.1372^{ab}	0.1368^{ab}
TF + clostripain	0.0814^{e}	0.0336^{f}	0.0122^{g}

Electrophoretic analysis of recombinant hirudin

Recombinant hirudin was purified from the unternatant fraction following Factor Xa cleavage of oil bodies from transformed seed. Purification was accomplished through anion exchange chromatography followed by reversed-phase chromatography. After each step, hirudin-containing fractions were identified using the anti-thrombin chromogenic assay. Two overlapping peaks of hirudin activity were obtained following reversed-phase chromatography. As shown in Fig. 8A, both peaks were comprised largely of a single molecular mass species indicating a relatively high degree of purity. While the apparent molecular mass of ca. 14 kDa observed for this protein is higher than the predicted mass of 6893 Da, it is identical to that seen on immunoblots

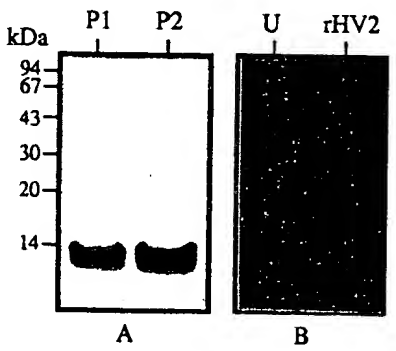

Fig. 8. Electrophoretic and immunoblot analysis of recombinant hirudin. A. *B. napus*-derived recombinant hirudin was purified and analyzed on a 16.5% tricine-SDS gel. Ca. 3.4 μg of peak 1 (P1) and 7.6 μg of peak 2 (P2) obtained upon reversed-phase chromatography were loaded in individual lanes. Protein was visualized via Coomassie blue-staining. B. An aliquot of the undernatant fraction (U) from transformed seed corresponding to ca. 80 μg of original oil body protein was separated on a 16.5% tricine-SDS gel along with 3 units of commercial, recombinant hirudin variant 2 (rHV2) (Sigma) derived from yeast. The resulting gel was electroblotted onto PVDF-P^{sq} membrane and probed with anti-hirudin antibodies.

(Fig. 8B) for both *B. napus* (U)- and yeast-derived recombinant hirudin variant 2 (rHV2). These results suggest that the difference between apparent and actual molecular mass is due to the aberrant behavior of hirudin on SDS gels. The precise differences between the two peaks of recombinant hirudin eluted from the reversed-phase column are unclear and are currently being investigated. However, the specific activity (measured through the use of an anti-thrombin chromogenic assay), was found to be identical for both peaks.

Discussion

We have demonstrated the efficacy of using oleosin gene fusions for the production of high-value recombinant proteins in plants. Transcripts of the oleosin-hirudin fusion gene accumulated in a seed-specific manner at the mid-cotyledonary stage of development. The corresponding fusion protein was detected in dry seeds and was localized primarily to the oil body phase of centrifuged seed proteins. Following proteolytic cleavage of the fusion protein, a fraction possessing anti-thrombin activity was recovered. While a rigorous quantitative analysis of expression awaits further work, levels of fusion protein observed on immunoblots (such as Fig. 5, panel B) lead us to suggest, tentatively, that they may represent up to 10% of endogenous oleosin protein levels and thus represent as much as 1% of the total seed protein. This level is comparable to that of other plant-based expression systems [12, 29, 35, 39] and could prove suitable for the cost-efficient production of recombinant proteins. Even higher levels of expression might be obtained through modifications of the truncated *Arabidopsis* promoter used in this study. Recently, it has been shown that this promoter contains regions responsible for both the up- and down-regulation of expression and that additional upstream sequences may further increase expression ten-fold [30].

One of the major problems associated with fusion protein expression systems is degradation or loss of the product due to improper folding and/or targeting. Hoffman and colleagues [13] showed that while a gene encoding a modified phaseolin protein was expressed correctly in transformed tobacco, the protein was subsequently degraded. Since the protein was observed to accumulate briefly in the Golgi apparatus, it was assumed that a failure in protein body targeting was responsible. Although the precise amino acid sequences involved in targeting oleosins to oil bodies are not yet known, analysis has identified conserved and variable regions within the oleosin protein [16]. Considerable variability has been observed between different species in both the N- and C-terminal regions of oleosin and thus these regions are the most likely to tolerate insertion of foreign sequences. Similar reasoning was employed successfully for the expression of Leu-enkephalin fused to an *Arabidopsis* 2S albumin [39]. However, because processing occurs at both the N- and C-terminal ends of this protein, it was necessary to insert the foreign peptide into an internal region, such that two separate proteolytic digestion steps were required to liberate the desired product. In addition to the increased complexity of processing, insertion into an internal region of a carrier protein severely limits the size of the foreign protein which could be accommodated [19]. Localization of the oleosin-hirudin fusion protein primarily to the oil body fraction demonstrates that targeting is not adversely affected and supports the suggestion that the C-terminal region of oleosin can tolerate considerable modification.

Recovery of active hirudin after proteolytic treatment of the oil body fraction indicates that proper folding of hirudin is able to occur and furthermore, that the protease recognition site is accessible while the fusion protein remains attached to the oil body. The pathway of hirudin folding has been studied extensively [4, 5] and has revealed that correct formation of the three disulfide bridges present in the native protein is necessary for full activity.

While it was not possible to assay for anti-thrombin activity in oil body preparations due their opacity, we believe that it is unlikely that hirudin is active while present in a fusion protein

configuration. This is based upon the fact that the N-terminal portion of mature hirudin is essential for binding and blocking the active site of thrombin [3]. In yeast expression systems, it has been shown that the failure to properly remove residues directing the secretion of recombinant hirudin resulted in its inactivation [9, 22]. In these studies an active product could be obtained after correct proteolytic processing of the signal sequence. Furthermore, when expressed in *E. coli*, the *B. napus* oleosin-hirudin fusion protein failed to exhibit anti-thrombin activity until it had been digested with protease to release hirudin (unpublished results). Based on these findings, it seems likely that steric hindrance from the oleosin portion of the fusion protein prevents the interaction between hirudin and thrombin such that oil body preparations from transformed plants would not possess anti-thrombin activity. The inactivity of the fusion protein has practical implications for the field production of recombinant hirudin in that the transgenic plants would be environmentally benign.

The cost associated with the purification of recombinant proteins is a significant factor in determining the feasibility of commercial production. The substantial level of purification achieved through flotation-separation of oil bodies demonstrates the utility of this technique for the production of recombinant proteins in plants. Our current estimates suggest that greater than 90% of contaminating seed proteins are removed following the simple flotation centrifugation procedure. The level of enrichment achieved is comparable to that achieved by the secretion of proteins in bacterial [2, 6] and yeast [31] expression systems. As a result, the further purification of hirudin to near-homogeneity was accomplished in only two chromatographic steps using standard methodology. The oleosin fusion protein technology is also amenable to large-scale production and can be readily integrated into existing agricultural processing procedures. Although oilseed rape is generally not wet milled, this process could be adapted from existing equipment and procedures routinely used for wet milling of other crops such as corn. Similarly, it has been shown [17] that equipment presently used in the dairy industry for the separation of milk products is suitable for the separation of oil bodies following aqueous extraction. Production of recombinant proteins in seeds also offers several distinct advantages over synthesis in bacteria and yeast. The low cost of seed-based expression systems contrasts with the expensive and labour-intensive operation of large-scale fermentation systems. There was no detectable decrease in fusion protein accumulation between seeds stored for over a year versus those newly harvested, indicative of the stability of seed proteins during storage. This means that there is no need to synchronize field production with demand for a given protein. Provided that expression levels are adequate and purification costs reasonable, seed-based expression systems should be very cost-efficient.

In conclusion, we have described a system for the general production and recovery of recombinant peptides synthesized as fusions to seed oil body proteins. The fidelity of this system has been demonstrated with the production of a functional pharmaceutical protein, hirudin. The system is flexible with respect to the different types of proteins it can accommodate and enables rapid and simple purification of the recombinant product. Furthermore, low costs of seed production and compatibility with existing agricultural processing procedures make it an attractive alternative to conventional bacterial and yeast fermentation systems.

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